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REVIEWS

Applications of the Ninhydrin Reaction for Analysis of Amino Acids, Peptides, and Proteins to Agricultural and Biomedical Sciences

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The reaction of ninhydrin with primary amino groups to form the purple dye now called Ruhemann's purple (RP) was discovered by Siegfried Ruhemann in 1910. In addition, imines such as pipecolic acid and proline, the guanidino group of arginine, the amide groups of asparagine, the indole ring of tryptophan, the sulfhydryl group of cysteine, amino groups of cytosine and guanine, and cyanide ions also react with ninhydrin to form various chromophores of analytical interest. Since its discovery, extensive efforts have been made to apply manual and automated ninhydrin reactions as well as ninhydrin spray reagents to the detection, isolation, and analysis of numerous compounds of interest across a broad spectrum of disciplines. These include agricultural, biochemical, clinical, environmental, food, forensic, histochemical, microbiological, medical, nutritional, plant, and protein sciences. This reaction is unique among chromogenic reactions in that at pH 5.5 it results in the formation of the same soluble chromophore by all primary amines which react, be they amines, amino acids, peptides, proteins, and even ammonia. Because the chromophore is not chemically bound to the protein or other insoluble material, it is not lost when the insoluble substrate is removed by centrifugation or filtration after the reaction is completed. The visible color of the chromophore is distinctive and is generally not affected by the yellow colors present in many food, plant, and tissue extracts. Adaptations of the classical ninhydrin reaction to specialized needs in analytical chemistry and biochemistry include the use of acid, alkaline, and fluorogenic ninhydrin reagents. To cross-fertilize information among several disciplines wherein an interest in the ninhydrin reaction has developed, and to enhance its utility, this review attempts to integrate and correlate the widely scattered literature on ninhydrin reactions of a variety of structurally different compounds. Specifically covered are the following aspects: historical perspective, chemistry and mechanisms, applications, and research needs. A better understanding of these multifaceted ninhydrin reactions provide a scientific basis for further improvements of this important analytical technique.

Keywords: Ninhydrin; Ruhemann's purple; amino acids; peptides; proteins; analysis; food chemistry; protein chemistry; clinical chemistry; histochemistry; forensic science; microbiology; medicine; nutrition; pharmacology; plant science; toxicology; proteolysis; cereal proteins; keratin proteins; milk proteins; soy proteins; whey proteins; protein-tannin complexes; cheeses; fruits; vegetables

INTRODUCTION

Ninhydrin reactions using manual and automated techniques as well as ninhydrin spray reagents are widely used to analyze and characterize amino acids, peptides, and proteins as well as numerous other ninhydrin-positive compounds in biomedical, clinical, food, forensic, histochemical, microbiological, nutritional, and plant studies. For several years, we have been studying the factors that influence the course, mechanisms, and applications of ninhydrin reactions for both fundamental understanding and to improve their analytic utility (*1*−*9*). We have shown that the mechanism of the reaction of ninhydrin hydrate with amino groups, producing the colored ninhydrin chromophore called Ruhemann's purple (RP) (λ_{max} 570 nm; $\epsilon = 22\,000$), can be explained in terms of polar and steric effects associated with the reactants. Our suggested mechanism based on kinetic and structural studies has been widely affirmed (*10*−*14*). Our own studies on the applications of the reaction include

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determining the biogenic amines histamine (*15*) and phenylethylamine (*16*); lysine (*7*, *17*−*22*); lysinoalanine (*23*, *24a*), histidinoalanine (*24b*,*c*), sulfur amino acids (*25*−*31*); tryptophan (*32*−*34*); internal standards for amino acid analysis (*35*, *36*); amino acids by ion-exchange chromatography (*3*, *28*, *31*, *36*−*39*); the amino group content of food proteins and flours (*7*), keratin proteins (*5*, *40*), and of fruits and vegetables (*9*); the proteolysis of cheeses (*8*) and soy proteins (*41*); and the extent of acetylation (*19*), glucosylation (*22*), and alkylations (*23*, *42*−*47*) of amino groups in amino acids, peptides, and proteins. The cheese and wool studies demonstrated that the ninhydrin assay is applicable to both soluble and insoluble materials. The value of the ninhydrin reaction to plant and food sciences is illustrated by reports that ninhydrin assays can measure protein components of plant protein-tannin complexes (*48*−*50*) and of whey added to milk (*51*). These aspects are examined in more detail below.

Although the ninhydrin reaction is used daily in thousands of laboratories throughout the earth and may very well be the most widely used organic reaction, several features associated with it appear to be anomalous. Thus, in many cases the amount of color formed is not always stoichiometric. It varies with the product analyzed and does not always correspond exactly to theory. Causes for this nonideal behavior need to be explained. Chemical aspects of ninhydrin reactions have been previously reviewed (*10*−*12*, *52*−*55*). The main objective of this review is to offer an integrated, critical overview of the chemical basis for reported applications of the reaction to agricultural, food, and biomedical sciences.

The widest possible interchange of ideas, viewpoints, and expertise is needed to transcend present limitations of the application of the ninhydrin reaction. Scientists from related disciplines need one another's improved methods. The range of topics covered includes a variety of specific and general interests.

HISTORICAL PERSPECTIVE

The following is a brief historical perspective on the discovery and uses of the ninhydrin reaction. In the course of studies of keto−enol tautomerism of cyclic ketones, Siegfried Ruhemann, working at the University Chemical Laboratory in Cambridge, England made a chance discovery in 1910 that was to revolutionize progress in the chemistry and biochemistry of amino acids, peptides, and proteins. In his own words: "The further study of triketohydrindene hydrate led to results which appear to be of great interest. It was found that a deep blue colour is produced on warming a mixture of aqueous solutions of this compound with aliphatic or an aliphatic−aromatic amine-which contains the amino group in the side chains. As shown below, this reactions has been successfully applied to a number of α-amino acids" (*56*).

This and related observations in his detailed studies on the chemistry of what became known as the ninhydrin reaction induced other investigators to explore the applicability of the new reaction to amino acid and protein chemistry, as exemplified by the following introductory statement from a 1911 paper by Emil Abderhalden and Hubert Schmidt originating from the Physiologisch Institut der Tierärtzliche Hochschule, Berlin, Germany: "Herr Prof. Ruhemann in Cambridge was kind enough to provide us with a sample of triketohydrindene hydrate, which he himself prepared and showed that it reacts at very high dilution with proteins, peptides, and amino acids forming a blue-colored dye, which has proved useful to demonstrate the presence of proteins and their degradation products in plants and animals. We studied the reaction of a large number of different compounds with this reagent in order to determine the extent to which the reaction is typical with different classes of compounds" (*57*). Of the 26 compounds investigated, 23 amino acids and 2 proteins produced the typical purple-blue color, whereas the color with proline was yellow. The initial observations on the ninhydrin reaction were followed by ensuing studies designed to extend the usefulness of the reaction (*56*, *58*−*70*).

A second seminal advance in the history of the ninhydrin reaction is the automation of the chromatography in 1948 by Sanford Moore and William H. Stein at Rockefeller University, enabling rapid assays of all amino acids in a protein hydrolysate at nanomole levels (*71*−*75*). Discussion of subsequent improvements in automated analysis with the aid of amino acid analyzers is beyond the scope of this paper. The following references offer an entry into the historical evolution of column chromatographic methods that utilize the ninhydrin reaction: (*3*, *37*, *38*, *76*−*87*). A computer program we created for the integration and computation of levels of amino acids in protein hydrolysates following chromatography (*3*) is still in wide use.

An outstanding third contribution by Dent (*88*), concurrent with automation, is the use of ninhydrin sprays to develop sixty ninhydrin-positive compounds on thin layer paper chromatograms. A sensitive paper-chromatographic analysis of amino acids in plants is described by Thompson and Morris (*89*). Such sprays are now widely used with paper and silica gel plates, as exemplified by applications described in the following selected references: α-amino acids (*90*); phosphoamino acids (*91*); cross-linked amino acids (*92*, *93*); biogenic amines (*94*); amino sugars (*95*); and domoic acid in shellfish (*96*). Investigators often use both TLC and column chromatography.

A fourth most important discovery is the observation that complexation of Ruhemann's purple (RP) with certain metal ions enhances the sensitivity of the analyses by allowing estimation of the resulting chromophore by fluorescent, luminescent, phosphorescent, and laser techniques. This modification, now widely used in forensic sciences to determine faint fingerprints, merits application to agricultural and biomedical sciences. The development of fluorogenic ninhydrin reagents (*97*, *98*) and their use in forensic science (*99*, *100*), as well as the application of ninhydrin reagents in environmental chemistry (*101*), food chemistry (*7*, *8*, *82*, *102*, *103*), clinical chemistry, microbiology, pharmacology, and toxicology (*104*−*108*), are active areas of current research.

CHEMISTRY AND MECHANISMS OF NINHYDRIN REACTIONS

α-Amino Acids. To help delineate the mechanisms of ninhydrin reactions, we studied reaction rates of α-amino acids with ninhydrin at 30 and 100 °C as a function of basicities and steric environments of amino groups (*1*). On the basis of the observed reactivity of amino acids at 30°, a linear free-energy equation was derived that makes it possible to calculate the influence on rates of separate polar and steric parameters associated with the amino acids. The rate-determining step in the ninhydrin reaction appears to involve a nucleophilic-type displacement of an OH group of ninhydrin hydrate by a nonprotonated amino group (**Figures 1** and **2**).

These and additional kinetic and mechanistic studies on the behavior of structurally different amino acids in ninhydrin reactions (*5*, *6*), which extended previous observations, indicated that the reaction of α-amino acids with ninhydrin entails two molecules of ninhydrin for each molecule of amino acid to form RP. Again, for α-amino acids, both polar and steric parameters are involved during the nucleophilic displacement of an OH

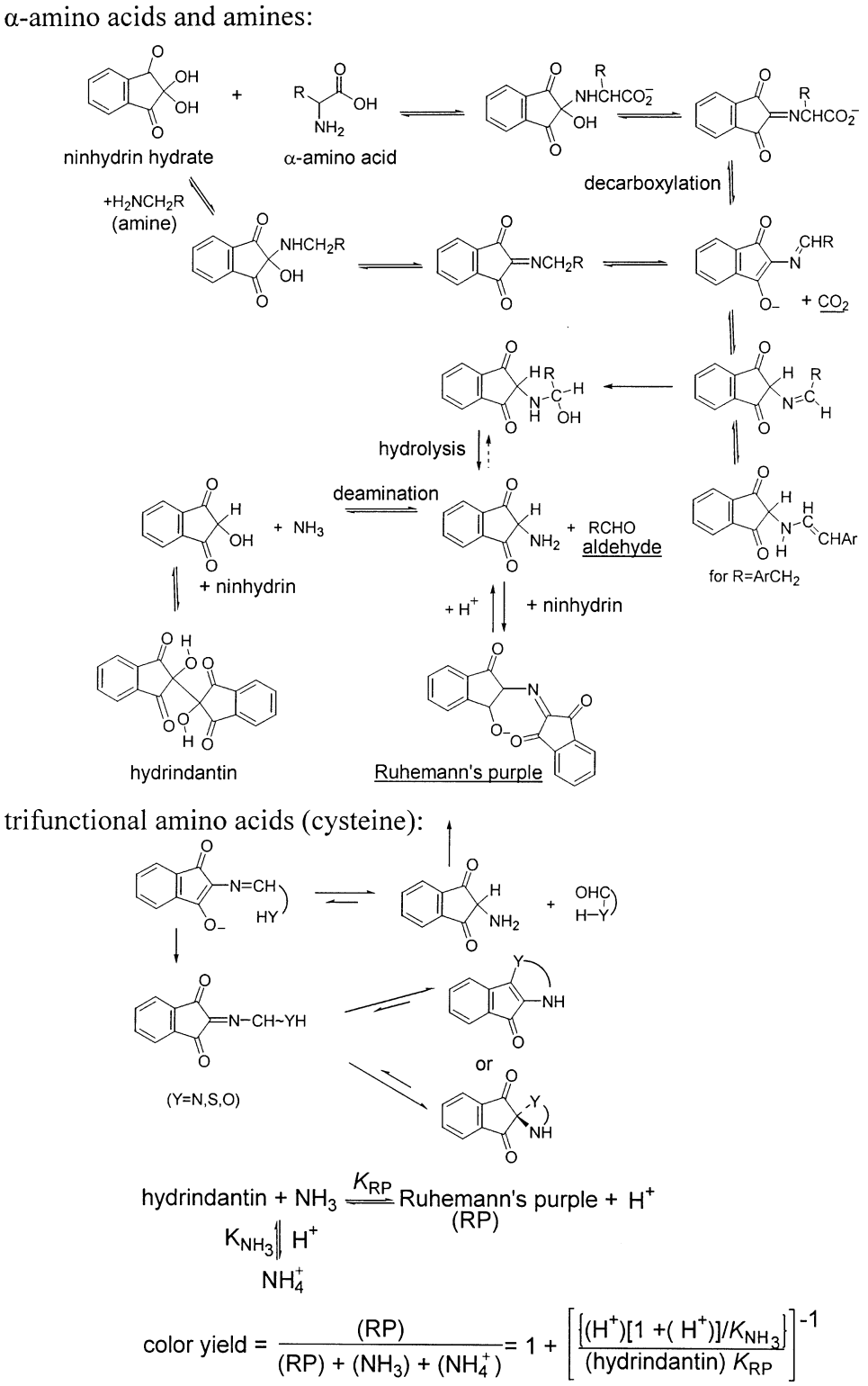

Figure 1. Mechanisms of reactions of α-amino acids, amines, and trifunctional amino acids with ninhydrin hydrate to form Ruhemann's purple and other products (*1*, *6*, *10–13*, *54*).

group of ninhydrin by an NH_2 group in the first step of the reaction. The results are consistent with the view that the first step is rate determining. It may be assumed that decarboxylation is not the rate-determining step because decarboxylation would be expected to be unimolecular and not subject to steric hindrance. The products of the reaction of α-amino acids with ninhydrin are CO_2, an aldehyde (RCHO), and RP (**Figure 1**).

The reaction mechanism of amines or amino acids that do not have the NH_2 α to the COOH group differs from that for α-amino acids shown in **Figure 1** in one major respect. The origin of the pair of electrons required for the indicated transformation is a carbon−hydrogen (C−H) bond and not a carboxyl group. The mechanism which predicts that amino compounds which have the amino group attached to a tertiary carbon atom should be ninhydrin negative has been unequivocally confirmed with studies with several model compounds (*1*).

Stoichiometry of Formation and Stability of Ruhemann's Purple. In some cases, RP does not correspond exactly to the

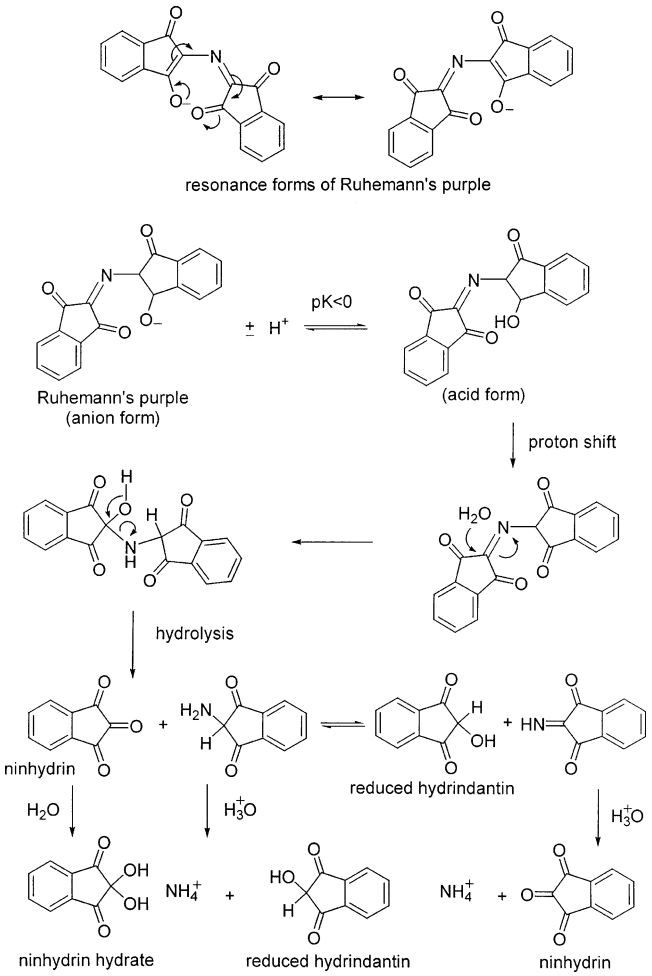

Figure 2. Electron delocalization and hydrolytic pathways of Ruhemann's purple in acid solution (*4*, *6*, *246*).

expected 1 stoichiometric equiv per amino group. Possible reasons for this apparent nonideal behavior include slow formation; side reactions; hydrolytic, oxidative, and photolytic instability; and interfering color. We (*6*) examined the origin and contributions of each of these factors. Two factors that affect the formation of RP are considered here.

(a) *Slow Reaction.* Some amines react with ninhydrin considerably more slowly than others so that a low color yield at one reaction time may sometimes be due simply to incomplete reaction. However, unless the reaction rate is impracticably small, measuring the color yield at several times will show whether the maximum has been reached.

(b) *Unfavorable Equilibrium.* Because it has several steps, the ninhydrin reaction has several points at which amine may be diverted from RP formation. Decarboxylation (loss of CO_2) and concurrent aldehyde formation are essentially irreversible, so any equilibrium before the last irreversible step (aldehyde formation) can only slow the rate of reaction.

Alternatively, acid-catalyzed hydrolysis of RP at low pH becomes fast relative to the time of reaction (**Figure 2**). The equilibrium is shifted by increasing H^+ and removing NH_3. Once this equilibrium becomes established, the color yield is no longer kinetically controlled. Quantitatively, the yield of RP should approach 100% at high pH and high hydrindantin concentration and approach zero at low hydrindantin concentration or in acid solution. In fact, however, ammonia and ninhydrin do react even in the absence of hydrindantin to form variable amounts of RP.

Table 1. Relative Color Yields Determined by the Manual Ninhydrin Reaction at 570 nm for Amino Acids, Peptides, and Diamino Acids (Molar Basis)

amino acids and peptides[a]	ninhydrin color (Leu equivalent)
alanine	1.03
alanyl-alanine	1.92
alanyl-alanyl-alanine	1.99
arginine	0.93
asparagine	0.78
aspartic Acid	0.90
cysteine	0
cystine	1.03
glutamic acid	0.96
glutamine	1.06
glycine	1.00
glycyl-glycine	0.81
glycyl-glycyl-glycine	0.78
glycyl-alanine	0.92
glycyl-alanyl-alanine	0.89
histidine	0.99
leucine	1.00
isoleucine	1.00
lysine	1.08
N^{α}-acetyl-lysine	0.98
N^{ϵ}-acetyl-lysine	0.95
γ-glutamyl-lysine	1.93
methionine	0.99
phenylalanine	0.81
proline	0
serine	0.81
threonine	0.86
tryptophan	0.88
tyrosine	0.93
valine	0.98
ammonia	0.86
diamino acids[b]	
1,3-diaminopropionic	0.12
2,4-diaminobutyric	0.71
2,5-diaminopentanoic (ornithine)	1.00
2,6-diaminohexanoic (lysine)	0.99
6-carboxy-2,6-diamino-hexanoic (diaminopimelic)	0.44
leucine	1.00

[a] Adapted from ref 8. [b] Adapted from ref 6.

Because the reaction *requires* reduction at some step, it seems that excess ninhydrin itself acts as a reducing agent.

Polyfunctional Amino Acids. Compounds that contain another nucleophilic group (amino, sulfhydryl) besides the α-amino group may form cyclic intermediates that are not converted to RP or converted only slowly (**Figure 1**). Thus, ornithine, lysine, and penta- and hexa-methylenediamine give color yields corresponding to only one of their two amino groups. Diamino acids such as lysine have low color yields with ninhydrin (**Tables 1** and **2**), presumably because the aminoaldehyde intermediate, produced by decarboxylative deamination, cyclizes to piperidine and thus prevents reaction of the second NH_2 group with ninhydrin (*31*). Lysine gives normal color yield when either of its two amino groups is blocked by acetylation. Cysteine reacts with ninhydrin forming a *spiro* derivative discussed below.

Although production of RP is generally taken as indicative for aliphatic primary amino groups, several cases are known in which aliphatic secondary amino groups (e.g., *N*-methylglycine, CH_3NHCH_2COOH) also give rise to the dye (*109*). The pH-bleaching profile of RP was examined in neutral, basic, acidic, and mixed aqueous-DMSO solvents (*5*, *6*). RP appears relatively stable in neutral and basic solution. It is possible to calculate an energy of activation in basic solution: $Ea = 6.15$ kcal per mole. This value is about one-half that in acid solution.

Table 2. Chromatographic Properties of Sulfur Amino Acids on an Amino Acid Analyzer[a]

cysteine and related cpds	elution time (min)	Leu color factors
cysteine methyl ester	70.82	0.02
L(–)cysteine	23.62	0.12
cysteamine	61.90	0.005
D(–)penicillamine	24.63	0.13
DL-penicillamine	24.85	0.13
γ-L-glutamyl-L-cysteine	54.4; 65.6 (double peak)	0.15
L-2-thiolhistidine	14.68	0.64
glutathione reduced	13.50	0.72
L-homocysteine thiolactone	70.68	0.82
DL-homocysteine thiolactone	70.75	0.82
D-homocysteine thiolactone	70.50	0.83
cysteic acid and related cpds		
hypotaurine (β-amino-ethanesulfinic)	7.32; 7.56 (double peak)	0.94
L-cysteic acid (cysteine sulfonic acid)	5.20	1.00
L-cysteine sulfinic acid	5.20	1.14
taurine (2-aminoethane-sulfonic acid)	7.73	1.02
DL-homocysteic acid	5.12	1.03
cystine and related diamino acids		
D-penicillamine disulfide	39.48	0.96
L-cystine dimethyl ester	84.9	0.28
L-cystine	33.45	0.96
D-cystine	34.05	0.94
DL-cystine	32.47	0.94
DL-lanthionine	19.88; 21.38 (double peak)	1.14
L- plus *meso*-lanthionine	20.18; 21.38 (double peak)	1.27
L-djenkolic acid	43.15	1.47
DL-*meso*-Homocystine	57.53	1.78
L-homocystine	59.23	1.80
D-homocystine	58.08	2.19
glutathione oxidized	13.07	1.54
DL(+)*allo*-cystathionine	43.23	1.06
L-lysine	69.60	1.04
methionine and related cpds		
L-methionine methyl ester	79.65	0.52
DL-methionine amide	44.67	0.89
L-methionine	44.37	0.97
DL-methionine sulfoximine	44.2	0.84
methionine sulfoxide	13.43; 15.83 (double peak)	1.01
methionine sulfone	15.25	1.06
L-methionylglycine	57.62	0.89
γ-glutamyl-5-methyl-L-cysteine	13.28	0.78
L-methionyl-L-phenylalanine	62.3	0.86
S-carboxymethyl-L-cysteine	12.98	0.91
S-carboxyethyl-L-cysteine	20.27	0.94
S-*n*-butyl-L-cysteine	48.43	0.94
S-methyl-L-cysteine	24.13	0.96
ethionine	46.45	1.02
S-β-(2-pyridylethyl)-L-cysteine, 2-PEC	66.77	0.73
S-β-(4-pyridylethyl)-L-cysteine, 4-PEC	69.07	0.82
S-β-(4-pyridylethyl)-L-penicillamine	75.92	0.84
S-β-(2-pyridylethyl)-penicillamine	71.12	0.92

[a] Adapted from refs 28 and 31.

Extrapolating to 100 °C at pH 5.5 gives an estimated hydrolysis rate of 1% per min.

Absorption Spectra of Ruhemann's Purple. As a result of our studies on the ninhydrin reactions in aqueous-DMSO solvents, it became apparent that the visible and NMR spectra of RP are strongly solvent-dependent (**Figures 3** and **4**). A study of the visible spectra of authentic RP in DMSO, formamide, DMF, and pyridine, as well as in mixed aqueous-nonaqueous solvents, revealed large differences in both the position of absorption of λ_{max} and ϵ values for the two bands (*4*). Both values of RP were a linear function of the composition of DMSO−H_2O solvent media. This informion facilitates predicting λ_{max} values of RP in different solvents. In nonaqueous aprotic solvents (i.e., DMSO and DMF), the λ_{max} should be near 605 mm; in nonaqueous aprotic solvents capable of undergoing acid−base equiibria (i.e., pyridine), near 550 nm; and in nonaqueous protic solvents (i.e., formamide), near 575 nm. The observed solvent effects need to be taken into account in quantitations of ninhydrin reactions in both nonaqueous and mixed aqueous−nonaqueous media. The possible use of NMR (**Figure 4**) to measure the concentration of RP has not been explored.

Other Reaction Products with Ninhydrin. There are many compounds that produce color with ninhydrin without giving RP. Imino acids (pipecolic acid, proline); aromatic amines (aniline), nucleic acids (cytosine, guanine); polyfunctional amino acids (arginine, asparagine, cysteine, tryptophan); and non-amino acids (fructose, levulinic acid, sialic acid, cyanide ions) condense with ninhydrin to form other "abnormal" products. These so-called "nonclassical" ninhydrin reactions are discussed below. **Figure 5** depicts the structures of the characterized ninhydrin derivatives of these compounds.

Arginine. The guanidino group of protein-bound arginine residues reacts with dicarbonyl compounds such as glyoxal (*22*, *110*) and ninhydrin hydrate to form cyclic adducts. Studies on the reaction of ribonuclease with ninhydrin at pH 8 and 25 °C helped assess the role of arginine residues in the structure and function of proteins (*111*). The author found that (a) ninhydrin modified arginine and lysine residues, resulting in inactivation of the enzyme; and (b) reversible acylation of the ϵ-NH_2 of lysine with citraconic anhydride followed by exposure to ninhydrin resulted in selective modification of arginine. A possible structure for the ninhydrin-arginine reaction product is shown in **Figure 5.**

Concurrent studies (*112*) revealed that (a) free cysteine and arginine reacted rapidly with ninhydrin at pH 8−9 at 37 °C; (b) reaction of free arginine with ninhydrin takes place in two stages, an initial rapid reaction followed by a 1000-fold slower reaction rate; (c) reaction of 2 mol of ninhydrin/mol trypsin resulted in the modification of 1.54 mol of guanidino groups/mol of trypsin without affecting tryptic activity; (d) the initial reaction between papain and ninhydrin resulted in the modification of 0.81 guanidino groups/mol of papain, suggesting that most of the arginine residues are not accessible to ninhydrin; and (e) the free SH group of papain was not modified by ninhydrin, suggesting that it is buried deep within the cleft of the active site of the enzyme. Ninhydrin can be used to titrate arginine residues of proteins. The use of a ninhydrin reagent to determine the content of guanidine derivatives in fruit trees is also noteworthy (*113*).

Asparagine. The enzyme L-asparaginase is widely used in medicine in the treatment of childhood acute lymphoblastic leukemia. The enzyme hydrolyzes L-asparagine to L-aspartic acid and ammonia, thus depleting free asparagine in blood. The molecular basis of the therapeutic effects is due to the fact that the growth of malignant cells is more dependent on an exogenous source of asparagine than is the growth of normal cells. Asparagine has also been shown to be a major precursor of potentially toxic acrylamide in processed food (*47*). A specific method for determining L-asparagine is based on its reaction with ethanolic ninhydrin solution at 37 °C to form a chromophore (**Figure 5**) absorbing in the UV at 340 nm (*114*). The assay was successfully applied to measure asparaginase and asparagines synthetase activities. The ninhydrin-asparagine chromophore is a stable intermediate in the RP production pathway of asparagines, analogous to that proposed for the enol-betaine structure of the proline-ninhydrin reaction product.

Related studies describe (a) experiments designed to minimize interference by other amino acids in the ninhydrin method for

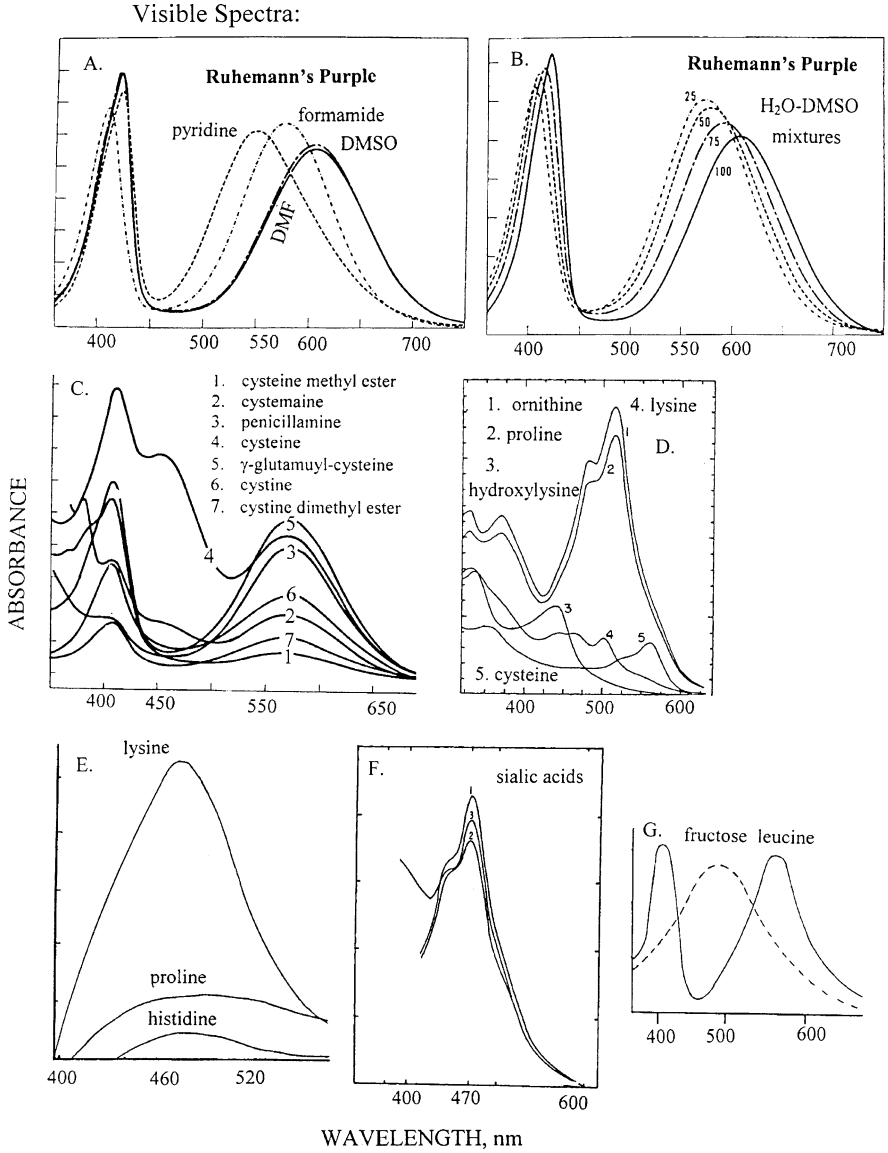

Figure 3. Absorption spectra: (A) Ruhemann's purple in different solvents, (B) Ruhemann's purple in DMSO–H_2O solutions (*4*), (C) Ruhemann's purple derived from sulfur amino acids (*31*), (D) ninhydrin-ferric chromophores (*124*), (E) acid-ninhydrin chromophores (*143*), (F) sialic (*N*-acetylneuraminic) acid–ninhydrin chromophores (*163*), (G) fructose-ninhydrin chromophore (*142*).

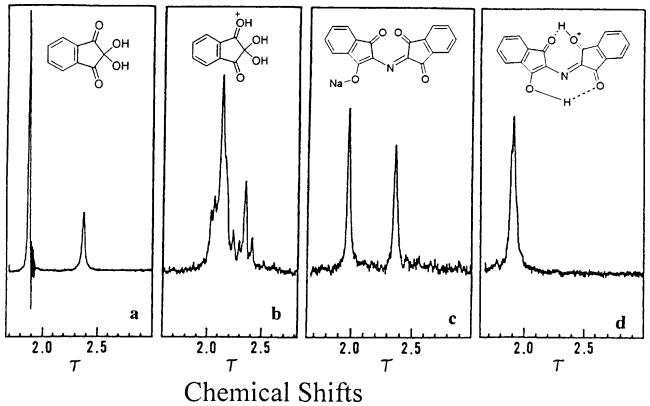

Figure 4. Nuclear magnetic resonance (NMR) spectra: (a) ninhydrin hydrate in DMSO, (b) ninhydrin hydrate in CF_3COOH, (c) Ruhemann's purple in DMSO, (d) Ruhemann's purple in CF_3COOH (*4*). The aromatic protons become equivalent on protonation in acid solution.

asparagine as a marker of freshness in asparagus (*115*, *116*), (b) a ninhydrin postcolumn derivatization method to quantitate *N*-methylasparagines formed posttranslationally in proteins (*117*), and (c) a method to attach oligosaccharide-asparagine derivatives to proteins (*118*). The presence of asparagine in the growth medium enhanced the resistance of human pathogens to inactivation at low pH (*119*).

Cysteine. The low color yield of cysteine can be explained as being due to a competitive nucleophilic displacement of the sulfhydryl and amino groups of cysteine on ninhydrin to yield as intermediate the spirane derivative (**Figure 5)** (*1*, *6*, *120*, *121*). L-Cysteine reacted with ninhydrin in acid solution consisting of 6 mL of acetic and 4 mL of concentrated HCl, forming a pink chromophore (λ_{max} 560 nm; $\epsilon = 2.8 \times 10^4$) (*122*). The method was used to measure the cysteine content of perchloric acid extracts of rat blood, brain, and liver. The cysteine SH groups of human and rat glucokinase isoforms, which are involved in pathogenesis of diabetes, were noncompetitively inhibited by ninhydrin and alloxan with a K_i value of 1 μM (*123*). The inhibition appears to be a reversible oxidation of the SH to S−S forms with concurrent reduction of ninhydrin, possibly to hydrindantin (see also Pharmacology and Toxicology).

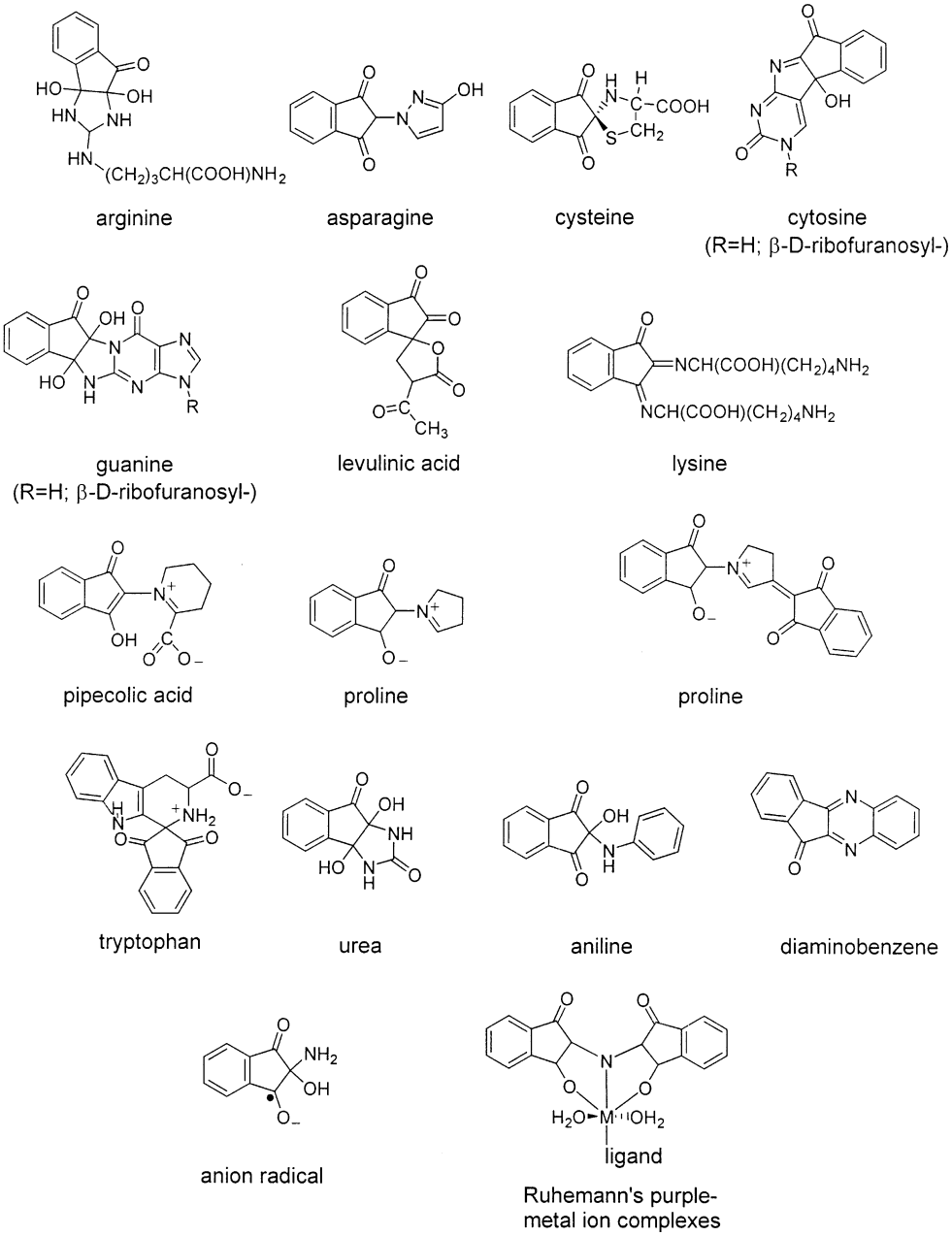

Figure 5. Structures of characterized ninhydrin derivatives: arginine (*111*), asparagine (*114*), cysteine (*121*), cytosine (*158*), guanine (*159*), levulinic acid (*146*, *160*), lysine (*124*, *125*), pipecolic acid (*107*), proline (*13*), tryptophan (*53*, *133*), urea (*70*), aniline (*2*), 1,2-diaminobenzene (*2*), ninhydrin radical anion (*12*, *164*, *165*), Ruhemann's purple-metal ion complexes (*11*, *213–215*).

Lysine. Lysine, ornithine, and proline reacted selectively with a pH 1.0 acid ninhydrin reagent (*114*) (**Figure 5**). These observations stimulated interest in applying this method to free lysine. The presence of ferric chloride in the reaction mixture resulted in a ninhydrin ferric reagent that reacted specifically with lysine at pH 1.0, forming a chromophore which absorbs at 470 nm, without interference by proline, ornithine, glycine, arginine, histidine, urea, ammonia, or sugars (*124*). DMSO was found to dissolve the ninhydrin−lysine−ferric complex, permitting analysis of lysine concentrations from 0.0625 to 0.5 mg. The method merits application to the determination of lysine in the industrial manufacture of lysine by lysine-producing microbes.

Other useful applications to lysine and derivatives include determination of (a) lysine with ninhydrin based on formation of the fluorescent ninhydrin-lysine derivative shown in **Figure 5** (*125*), (b) unreacted lysine by ninhydrin to facilitate isolation of a lysine-glucose nonenzymatic browning product (*126*), (c) γ-glutamyllysine (*21*, *93*), (c) lysinoalanine in food proteins (*23a*,*b*), (d) galacturonoyl-L-lysine amides in plant cell walls (*127*), (e) cross-linked and glycated amino acids in collagen (*128*), (f) hydroxylysine glycosides in urine (*129*), (g) the biosynthesis of lysine in bacteria (*130*), and (h) N^6-(1-carboxyethyl)lysine in *Streptocococcus lactis* cultures (*131*). See also section below on Ninhydrin-Reactive Lysine in Proteins.

Tryptophan. The amino acid tryptophan is of paramount importance in nutrition, physiology, and food science. Analytical methods for tryptophan have been extensively studied, but with limited success, primarily because of interference by carbohydrates and because the indole ring of tryptophan is degraded under acid conditions used for protein hydrolysis. Because tryptophan cannot be analyzed along with the other amino acids by standard amino acid analysis techniques, numerous attempts have been made to devise modified hydrolytic methods that do

Table 3. Tryptophan Content of Proteins and Flours Determined by an Acid Ninhydrin Assay without Hydrolysis[a]

protein source	N (%)	tryptophan (g/16 g N)
casein	13.6	1.70
lysozyme	16.2	7.66
soy protein	14.0	1.36
barley flour	1.26	1.55 (1.27)[b]
beef, minced	13.6	1.25 (1.40)
corn flour	1.46	1.85 (1.28)
cottonseed flour	10.0	1.37
lima bean flour	3.32	1.42 (1.31)
oat flour	2.56	1.68 (1.33)
rice flour	0.98	1.72 (1.37)
soybean flour	8.32	1.43 (1.33)

[a] Adapted from ref *33*. [b] Values in parentheses are for protein extracts of flours. Extractions were carried out by the method of Concon (*251*).

not destroy tryptophan. Hydrolysis by HC1 in the presence of thioglycollic acid, as well as hydrolysis by mercaptoethanesulfonic, methanesulfonic, *p*-toluenesulfonic acids, NaOH or $Ba(OH)_2$ minimize losses of tryptophan; for review, see (*34*).

To overcome problems with the liberation of tryptophan from peptide bonds by acid and alkaline hydrolysis, a method was proposed for the determination of free and protein-bound tryptophan after reaction with ninhydrin in a mixture of formic and hydrochloric acid for 10 min at 100 °C (*132*). Under these conditions, tryptophan is transformed to a yellow product (λ_{max} 390 nm; ϵ7120). The method was used to measure the tryptophan content of the rat brain during development as well as the following intact proteins: BSA, lysozyme, insulin, ribonuclease, and histone. At a pH of about 1.0, tryptophan reacts with ninhydrin to form the yellow product 2,3,4,5-tetrahydro-β-carboline (*133*) (**Figure 5**). It is likely that this compound is responsible for the absorbance of the acid−ninhydrin product mentioned above.

Zahnley and Davis (*134*) noted that colorimetric determination of tryptophan in intact proteins by the acidic ninhydrin method gave high values for proteins having high tyrosine/tryptophan ratios. To avoid such erroneous results, these authors developed a successful technique to correct for the interference by tyrosine absorption. The method was successfully used to measure the tryptophan content of chicken ovoinhibitor, which contains 17 tyrosine residues per molecule. The acid ninhydrin method was successfully adapted to protein extracts of beans, maize, and wheat (*135*). The obtained tryptophan values, corrected for tyrosine absorption, were similar to those reported by other methods for the same commodities.

To assess the utility of these previous reported methods, we evaluated the nonhydrolytic acid−ninhydrin method (**Table 3**) and six hydrolytic-ion exchange chromatographic methods with three proteins and a number of carbohydrate-containing food products (*33*). For the acid−ninhydrin method, a standard curve for lysozyme was used as a reference to calculate the tryptophan content of the other materials tested. The results show that for carbohydrate-containing foods, (a) the thioglycollic and organic acid methods appear ineffective, (b) basic hydrolysis appears preferable over hydrolysis in acids, and (c) the acid−ninhydrin method gave good reproducibility with a wide range of commonly used food products. Kinetic studies on the reaction of an acid−ninhydrin reagent with tryptophan in pure proteins (bovine serum albumin, human serum albumin, casein, α-chymotrypsin, lysozyme, trypsin) and protein-containing foods (barley, maize, rye, soybean meal, sunflower, fish, and meat meals) further demonstrated the usefulness of the non-hydrolytic method for tryptophan (*136*, *137*).

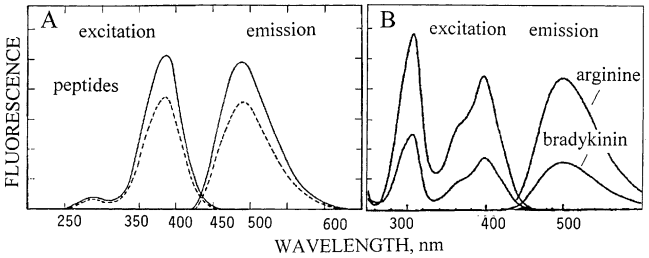

Figure 6. Fluorescent ninhydrin chromophores: (A) amino acids and peptides (*156*, *157*), (B) free arginine and arginine in bradykinin (*98*).

Modification of SH groups of cysteine residues of proteins by vinylpyridine prior to hydrolysis with mercaptoethanesulfonic acid vapors resulted in good recoveries of tryptophan from lysozyme and myoglobin by ninhydrin analysis on an amino acid analyzer (*138*). This approach to prevent destruction of tryptophan by cysteine residues merits further study with proteins in complex foods. The mechanism of reaction of tryptophan with ninhydrin in micelles at pH 5 to form RP is similar to that in a homogeneous aqueous medium (*139*, *140*).

N−Alkyl α-Amino Acids. In the course of a study on the N- and C-alkylation of peptides and proteins in DMSO, we found that the ninhydrin color yield relative to that of Leu for *N*-methylglycine and N-benzylglycine was about one-half that found for glycine, in contrast to negligible color for *N*-acetylglycine, *N*-methyl-α-alanine, and N-benzyl-α-alanine (*109*). The reason for the unexpected results with the N-alkylglycines is not known. We also found that ϵ-*N*-methyl-lysine and π- and τ-*N*-methyl-histidines are not well utilized by mice as nutritional sources of the unsubstituted amino acids (*141a*). See also ref *141b*.

Other Amino Acids. Ninhydrin assays are extensively used in studies on the formation, isolation, and characterization of so-called free ninhydrin-positive compounds from plant, bacterial, animal, and human sources. The following are some examples from the literature: (a) proline (*142*, *143*) (**Figure 5**), (b) diaminopimelic acid (*144*, *145*) (**Table 1**), (c) levulinic acid (*146*) (**Figure 5**), (d) ornithine (*147*), (e) sialic acid and other hexosamines in glycoproteins (*148*, *149*) (**Figure 3**), (f) methyl-histidines (*150*, *151*), (g) N^{δ}-acetyl-1-ornithine in mushrooms (*152*), (h) amino acids and peptides in the intestine (*153*), (i) amino acids in human oral fluids (*154*), and (j) pipecolic acid in biological samples (*107*, *155*) (**Figure 5**).

Fluorescence Products. A reaction mixture of ninhydrin, an aldehyde such as phenylacetaldehyde, and a primary amine yields a ternary, highly fluorescent complex of unknown structure (**Figure 6A**). This reaction is the basis for an automated fluorescent ninhydrin procedure for detecting amino acids and peptides, which is 10−100 times more sensitive than the colorimetric ninhydrin method (*156*, *157*). The method appears useful for detecting physiologically active peptides in biological tissues (**Figure 6B**) (see also section on Arginine).

Aromatic Amines. The reaction of ninhydrin with aromatic amines such as aniline does not result in the formation of RP. Aromatic NH_2 groups react with ninhydrin in nucelophilic displacements to form products having the structure shown in **Figure 5**. With diamino compounds such as *o*-phenylenediamine, ninhydrin forms the quinoxaline derivative (**Figure 5**). Elsewhere, I discuss in detail the spectral properties and mechanism of formation of the reaction products from primary aromatic amines and ninhydrin (*2*). Analogous polycyclic adducts are formed from the reaction of ninhydrin with guanine,

guanosine, cytosine, cytidine, and cytidine nucleotides (*158*, *159*) (**Figure 5**). These reactions need to be taken into account in applications of the ninhydrin reaction to nucleic acid-containing substrates.

Non-nitrogenous Compounds. Several investigators observed anomalous "ninhydrin-positive" reaction products of nitrogen-free aldehydes, ketones, keto acids, and monosaccharides (*146*, *160*, *161*). A proposed structure for the colorless ninhydrin-levulinic acid reaction product is shown in **Figure 5**. Other studies showed that an acidic ninhydrin reagent mentioned earlier was useful for measuring sialic acids both free and in sialoglycoproteins (*162*, *163*). The structure of sialic acid (*N*-acetylneuraminic acid) contains an acetylated NH_2 group. These observations should be taken into account in interpreting results from ninhydrin reactions.

Free Radical Products. Ninhydrin reacts with amino acids to produce free radicals characteristic of various amino acids as well as of N-terminal amino acids in peptides and proteins (*55*, *164*, *165*) (**Figure 5**). Related studies indicate that ninhydrin also participates in solid-state cascade reactions with proline (*166*) and that the photochemistry of ninhydrin is solvent-dependent (*167*).

Chromatographic Properties of Sulfur Amino Acids. One of the most common and accurate ways of determining amino acids in proteins and physiological fluids is by automated ion exchange chromatography. When this technique is used with proteins, the sample must be hydrolyzed before it is applied to the column. Hydrolysis introduces many problems because the sulfur amino acids are partly destroyed by acid treatment at high temperatures and interact with other components commonly present. For these reasons, direct assay of cysteine and in particular cystine gives low values. Cysteine is subject to oxidation during acid hydrolysis and chromatography, while cystine is racemized to the meso and DL forms. Both cysteine and cystine suffer losses by reaction with tryptophan, carbohydrates, and other substances commonly present. For these reasons, attempts have been made to change cystine and cysteine residues of proteins quantitatively to derivatives that are stable to acid hydrolysis and can be eluted as discrete peaks, so that they can be determined in the usual way with the other amino acids. With physiological fluids (e.g., urine, plasma), in which amino acids are in the free state, the main problem is to resolve the more than fifty ninhydrin-positive compounds that may be present. **Table 2** summarizes chromatographic properties of various sulfur amino acids as observed in our laboratory. Below are described some of the problems associated with specific sulfur amino acids mostly based on our studies (*26*−*28*, *31*, *83*).

Cystine. Cystine peaks are skewed because of racemization during hydrolysis, producing *meso*-cystine and DL-cystine (*28*, *30*). Normally, cystine is partly destroyed when heated with 6 N HC1. Nevertheless, when the protein is pure and tryptophan concentration is minimal, almost complete recovery can be obtained if oxygen is excluded during hydrolysis. More commonly, when appreciable amounts of carbohydrate or tryptophan are present, these prevent satisfactory recovery of cystine. To avoid this difficulty, much work has been done to convert cystine to a stable derivative (e.g., preoxidizing it to cysteic acid, releasing it by acid hydrolysis, or alternatively, reducing it to form cysteine and further treating the cysteine-containing protein to form an altered, stable derivative). Such treatments require one or more extra steps besides the usual hydrolysis. Accurate determination of cystine is difficult. The most widely used method for cystine uses performic acid oxidation to cysteic acid.

Cysteic Acid. Cysteic acid in performic-acid-oxidized samples originates from cystine, cysteine, and cysteic acid present before analysis (*26*, *83*). Cysteic acid is eluted as a discrete peak, separate from other constituents. With proper allowance for cysteic acid in an unoxidized sample, cysteine plus cystine in a protein can be determined as cysteic acid. Adding a reducing agent, such as HBr, to destroy excess performic acid results in a 94% recovery of cysteic acid and in a 100% recovery of methionine sulfone. This modification therefore permits simultaneous determination of methionine and cysteine plus cystine residues.

S-Carboxymethylcysteine. Protein SH groups can be alkylated with iodoacetamide. Hydrolysis of the modified proteins yields S-carboxymethylcysteine (*30*). This cysteine derivative appears as a well-resolved peak before aspartic acid. If protein disulfide bonds are first reduced, this procedure can also be used to estimate the cystine content separately.

S-Carboxyethylcysteine. Acrylamide, acrylonitrile, or methyl acrylate can modify SH groups in proteins by converting them to derivatives that form S-carboxyethylcysteine on hydrolysis. This cysteine derivative appears as a well-resolved peak (*168*).

S-β-(2-Pyridylethylcysteine). We evaluated 2-vinylpyridine as a reagent for selective chemical modification of SH groups (*27*−*29*). Cystine residues were quantitatively transformed and are accounted for in hydrolysates as the expected S-β-(2-pyridyl)ethyl-L-cysteine. The new amino acid survived unchanged up to 72 h under hydrolytic conditions.

S-β-(4-Pyridylethylcysteine). We also reduced S−S bonds of several proteins and seed meals (whole gluten, bovine serum albumin, lysozyme, β-lactoglobulin, and wheat and soy flours) to SH groups. The SH groups were then treated with equimolar amounts of 4-vinylpyridine. The cysteine residues were quantitatively modified to S-β-(4-pyridylethyl)-L-cysteine (4-PEC) residues. 4-PEC is stable to acid protein hydrolysis (*26*, *30*).

These PEC procedures have several advantages for determining cystine in proteins: (a) The cystine content can be estimated by two independent techniques, ion-exchange chromatography of hydrolyzed proteins and UV spectroscopy of hydrolyzed and unhydrolyzed proteins. (b) The method can be readily adapted to measure the degree of reduction in partly disulfide bond-reduced proteins.

Homocysteine. Analysis of cystine, homocystine, and mixed cystine-homocystine disulfides by ion-exchange chromatography with ninhydrin postcolumn derivatization is a widely used tool in studies of diagnosis and management of inborn errors of transsulfuration pathways. The automated assay can be used to measure total plasma homocysteine as an indicator of inherited defects of homocysteine metabolism as well as a marker of increased risk of heart disease. An improved assay for homocysteine was devised by carrying out the reduction of the mixed disulfides by dithiothreitol immediately after obtaining the plasma sample (*169*).

Methionine and Derivatives. Methionine is unstable to acid hydrolysis. Therefore, the conventional method is to oxidize these compounds to methionine sulfone by performic oxidation before HC1 hydrolysis (*83*). The methionine sulfone that is obtained represents the total of methionine, methionine sulfoxide, and methionine sulfone in the original sample. A nearly quantitative yield is generally obtained. It is relevant to note that SH groups of cysteine can reduce methionine sulfoxide to methionine (*170*).

Lanthionine. Lanthionine, a natural constituent of the peptide antibiotic subtilin, is also formed in proteins exposed to heat and high pH (*24*, *27*, *28*). It emerges either as a single or double peak depending on whether the sample contains the meso isomer in addition to optically active forms. DL and meso forms appear as two peaks between serine and proline.

Other Sulfur Amino Acids. Many other sulfur amino acids, both natural and synthetic, have been successfully separated on ion exchange columns, while still others have not. These include cystathionine, djenkolic acid, glutathione, β-2-thienylalanine, α-2-thiolhistidine, and taurine. Onions contain many interesting sulfur amino acids and peptides which have been separated on ion exchange columns and identified (*171*). S-Methylmethionine found in vegetables can be separated from other amino acids in plant extracts by changing the pH used with the basic column. Cystine, cysteine−penicillamine mixed disulfide, and penicillamine disulfide have been analyzed in the presence of other plasma amino acid (*83*).

Ninhydrin Color Factors. Examination of the extent of production of RP under automated conditions of a single-column amino acid analyzer by several classes of sulfur-containing amino acids (*31*) has revealed a wide variation in the color factors relative to Leu (**Table 2**). These ranged from 0.02 for the methyl ester of cysteine to 2.19 for D-homocystine. The color yield of cysteine is about one-eighth that of Leu. Esterification of the COOH group of cysteine results in further reduction of the color yield to 0.02. The color yields of 2-thiolhistidine homocysteine thiolactone, and reduced glutathione approach "normal" values

Lactonization of the SH group to form homocysteine thiolactone prevents the SH group in this compound from reacting with ninhydrin. The color yield is correspondingly high and suggests that the thiolactone is retained during the ninhydrin reaction. Homocysteine thiolactone with a color yield of 0.8 behaves as a normal amine in the ninhydrin reaction.

Comparison of color factors obtained by the manual ninhydrin method for L-Cys (0.156), the dipeptide α-L-Glu-L-Cys (0.348), and the tripeptide γ-L-Glu-L-Cys-Gly (GSH, 0.909) suggests that the progressive increase in these values may be due to the increase in distance between the NH_2 and SH groups in the three compounds, which minimizes the extent of the ring formation in the higher homologues. This trend is less pronounced in the automated procedure that gave the following color yields for the three compounds: 0.12, 0.15, and 0.72, respectively.

An additional factor operates in the case of cysteine methyl ester (a potent inhibitor of polyphenol oxidase (*172*)), which produces very little color in the automated ninhydrin reaction. The mechanism of the ninhydrin reaction (**Figure 1)** shows that the carboxyl group of an amino acid is decarboxylated in reacting with ninhydrin. Because the ester group cannot undergo decarboxylation, its low color yield may perhaps arise from the energetically unfavorable carbon−hydrogen cleavage undergone by amines with no carboxyl groups on the same carbon atom in reacting with ninhydrin.

The color factors range from 0.29 for cystine ester to 2.19 for D-homocystine. Because all these compounds have two amino groups per molecule, the expected color yields should be near 2. Again, the question arises as to the reasons for the wide range of observed color (**Table 1**). One possible rationalization is that an amino acid undergoes decarboxylative deamination in the course of reacting with ninhydrin to form an aldehyde. Because aldehydes also readily combine with amines, the resulting aldehyde probably competes with amino groups for ninhydrin. If such side reactions are indeed the cause for lower than expected color yields with some diamino acids, this side pathway would be predicted to operate mainly with those diamino acids that can form stable six- or seven-member rings. It is noteworthy that lanthionine, which might form a six-member ring, and cystine and lysine, which might form seven-member rings, all produce about one-half the expected color for diamino acid. In contrast, homocystine and djenkolic acid, which are also diamino acids, but which would form less readily synthesized eight- or nine-member rings in the side reaction, produce much higher color yields, supporting the cited hypothesis for the behavior of diamino acids.

Examination of chromatographic properties of some S-alkyl amino acids (methionine and cysteine derivatives) (*31*) reveals that with the exception of methionine methyl ester, nearly all exhibit near "normal" or expected color factors. Generally, peptide bond formation appears to decrease the amount of the ninhydrin color produced, as illustrated with the Leu color factors for Met (0.97) and the dipeptides L-Met-Gly (0.89) and L-Met-L-Phe (0.86). The low color value observed with methionine methyl ester (0.52) compared to methionine follows the general trend already mentioned for amino acid esters. However, the nearly "normal" value for methionine−amide (0.89) is surprising, because we would have expected it to behave like the methyl ester. In the case of the amide, some hydrolysis occurs to liberate ammonia, which also participates in the ninhydrin reaction and thus contributes to the purple color.

Elution Times. Examination of elution times (*28*, *31*, *83*) for various compounds (**Table 2**) with respect to their structure reveals general trends: (a) Esterifying the carboxyl group results in a dramatic shift in elution time, as exemplified by the 23.5-min elution time for cysteine and 70.5-min time for the methyl ester. Similar shifts are noted with cystine (33.3 min) and cystine methyl ester (84.9 min), and for methionine (44.2 min) and methionine ester (79.4 min). (b) The 70-min elution time for homocysteine thiolactone suggests that the compound exists completely as the lactone rather than as the open-chain homocysteine form, which would be expected to elute near cysteine in the region 23−25 min, because homocysteine differs from cysteine in having only one extra CH_2 group. (c) Substituting two methyl groups on the β-carbon atom of cysteine, to which the SH group is attached, results in 1-min-longer elution time for penicillamine. (d) Oxidizing the SH group of cysteine to an SO_2H (sulfinic acid) or to an SO_3H (sulfonic acid) results in decreased elution time from 23 to 5 min. (e) Trends in elution times of cystine and related diamino acids show that the number of sulfur atoms and increased chain length both tend to slow elution. For example, the additional two methylene groups in homocystine cause a shift of about 25 min in elution time compared to cystine. (f) The carboxyl group in carboxyethylcysteine decreases elution time of cysteine. Alkyl side chains increase it. The shift is quite pronounced for aromatic residues. Thus, 2-PEC elutes at 69 min compared to 23 min for cysteine. The observed trends in elution times should be useful for predicting color yields and elution positions of new, structurally related amino acids.

Biogenic Amines. The biogenic amine histamine is formed from free histidine by action of histidine decarboxylase. Our studies (*15*) showed that an assay for histamine along with all other amino acids can be carried out on a single-column amino acid analyzer with postcolumn ninhydrin derivatization. Histamine appears as a well-resolved peak, as determined with histamine added to a standard mixture of amino acids, cohy-

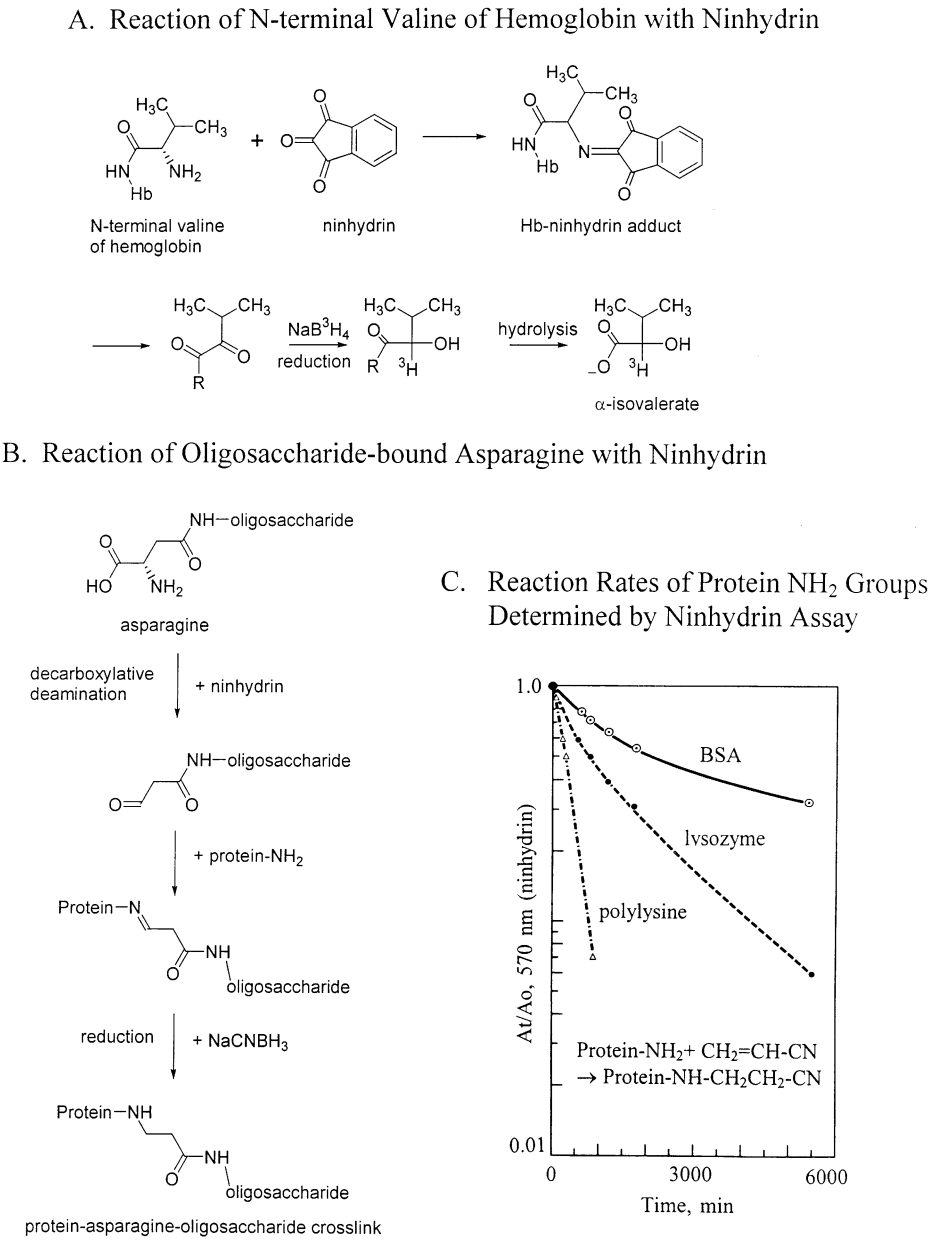

Figure 7. Protein-ninhydrin reactions: (A) N-terminal valine of hemoglobin (*175*), (B) asparagine attached to an oligosaccharide (*118*), (C) reaction rates of bovine serum albumin, lysozyme, and polylysine with acrylonitrile determined with ninhydrin (*42*).

drolyzed with casein, added to mouse serum and urine, and in a spoiled tuna sample.

In a related study (*16*), we developed a chromatographic method to measure both the biogenic amine phenylethylamine and its dehydroalanine adduct, phenylethylaminoalanine. These methods merit study with biogenic amines and their derivatives formed in food (*173*).

PROTEIN-NINHYDRIN REACTIONS

General Aspects. The ninhydrin assay of amino groups in proteins has been less satisfactory than procedures for analyzing amino acids. Because protein amino groups reacting with ninhydrin do not always produce the theoretical yield of RP, conditions need to be found to optimize the ninhydrin reaction of amino groups in food and other proteins. A pyridine−phenol−water ninhydrin reagent gave a 65−70% color yield (Leu = 100) for nonterminal lysine residue of several peptides and proteins (*174*). We carried out detailed studies to find the best conditions for assay of keratin proteins (wool, human hair) (*5*, *6*). The best solvent system was found to be a mixture of DMSO and water in the range 40−60% vol. The stability of both the ninhydrin reagent and chromophore produced (RP) are enhanced by DMSO.

Hemoglobin. Exposure of hemoglobin in pure form or in red cells to ninhydrin under physiological conditions (pH 7.4, 37 °C) for 2 h resulted in deamination (Strecker degradation) of the ϵ-NH_2 of lysine and the α-NH_2 group of N-terminal valine (*175*) (**Figure 7A**). Increasing the amount of modification with ninhydrin decreased the oxygen affinity of the hemoglobin. Although the effect of the deamination of hemoglobin on sickling of red blood cells is not known, alkylation of the α-NH_2 group of N-terminal valine by acrylamide was found to be a useful marker of the extent of human exposure to this toxic vinyl compound (*47*).

Protein−Asparagine−Oligosaccharide Cross-Links. Asparagine attached to an oligosaccharide was deaminated and decarboxylated by ninhydrin to an aldehyde side chain. Reaction of the aldehyde moiety with a protein amino group resulted in

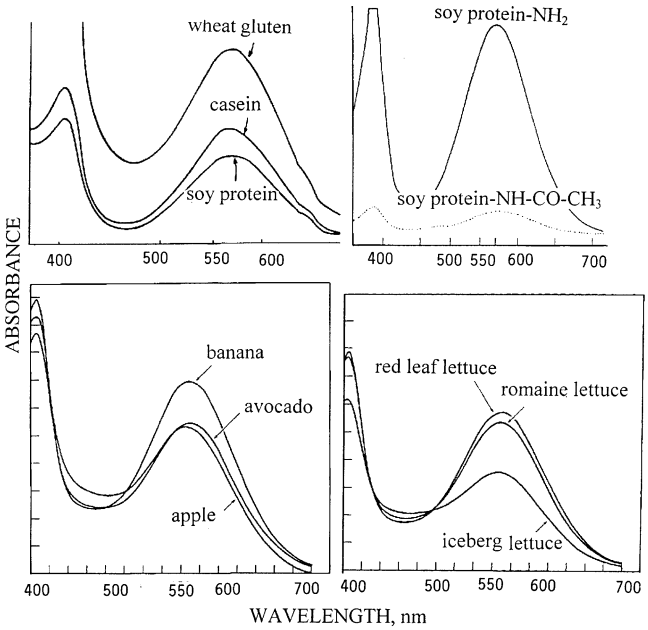

Figure 8. Absorption spectra of Ruhemann's purple derived from the ninhydrin reaction of food proteins and fruits and vegetables (*7*).

the formation of Schiff base (*118*) (**Figure 7B**). Reduction of the double bond of the latter by $NaCNBH_3$ (a reagent we introduced into protein chemistry (*176*, *177*)) gave a stable cross-linked protein-oligosaccharide.

Modification of Protein Amino Groups. Figure 7C illustrates the application of the ninhydrin reaction to follow the extent of alkylation by acrylonitrile of NH_2 groups in bovine serum albumin, lysozyme, and polylysine. The decrease in the ninhydrin chromophore is directly related to the extent of modification. **Figure 8** shows a similar decrease on acetylating of soy protein NH_2 groups. Below are described related studies designed to demonstrate the applicability of the ninhydrin reaction for analysis of so-called chemically and presumably nutritionally available lysine in food proteins.

Ninhydrin-Reactive Lysine of Food Proteins. The ϵ-NH_2 group of protein-bound lysine reacts with food ingredients under the influence of heat and high pH (*18*). To enhance the usefulness of the ninhydrin reaction for proteins, we (*7*, *8*) carried out studies designed to (a) test a ninhydrin reagent solution that would produce optimum amounts of the ninhydrin chromophore in food proteins; (b) establish the reproducibility of the reaction with several food proteins of varied lysine content; and (c) establish the extent of variation of color yield per lysine residue in structurally diverse food proteins in terms of Leu equivalents, a widely used measure of ninhydrin color yields, because the molar absorption of RP derived from Leu is identical to the corresponding value for authentic RP. Absorption spectra and the linear concentration response of RP produced by three food proteins, wheat gluten, casein, and soybean protein, are shown in **Figures 8** and **9**.

Our studies confirm the advantage of the lithium acetate−DMSO solution over the commonly used sodium acetate buffer in the ninhydrin reaction of food proteins. Compared to other combinations tested (water−DMSO, water−ethanol, water−acetonitrile, and water−acetone mixtures), the lithium acetate−DMSO combination was found most suitable for keeping food proteins and buffer salts in solution during the reaction, for stabilizing the ninhydrin chromophore for 4 days or longer when kept in a refrigerator, and for giving excellent reproducibility with a range of food proteins. The recommended ninhydrin reagent was prepared by dissolving 2 g of ninhydrin and 0.3 g of hydrindantin in 75 mL of DMSO, to which was added 25 mL of 4 M lithium acetate buffer. The pH was then adjusted to 5.2. The reagent could be stored up to two weeks in a refrigerator.

Table 4. Ninhydrin-Reactive ϵ-NH_2 Groups of Lysine Residues in Proteins[a]

protein	A^b due to ϵ-NH_2/ mg dry sample (X)	theor. A due to Lys ϵ-NH_2/ mg dry sample (Y)	Leu equivs/ Lys (X/Y)[c]
lactalbumin	0.76	1.19	0.64
casein	0.85	1.20	0.70
soybean protein	0.63	0.87	0.72
lysozyme	0.62	0.82	0.75
hemoglobin	0.98	1.30	0.76
bovine trypsin inhibitor	0.76	0.85	0.90
wheat gluten	0.21	0.23	0.93
trypsin	0.82	0.88	0.94
bovine serum albumin	1.13	1.03	1.10

[a] Adapted from ref 7. [b] A = absorbance. [c] Ninhydrin-reactive Lys (mmol/100 g) = (A/mg) × (volume of ninhyddrin solution)/(molar A of Leu) × (Leu equivalent/Lys) × 10^5.

Table 5. Relationship between Nitrogen (Protein) and Lysine Content and Ninhydrin Color Yields of Food Flours[a]

food flour	N[b] (%)	lysine g/100 g	lysine mmol/100 g	A at 570 nm/mg dry sample
rice	1.13	0.317	2.17	0.055
wheat	1.87	0.283	1.94	0.073
barley	1.55	0.390	2.69	0.084
high lysine corn	1.48	0.420	2.87	0.132
high protein rice	4.36	0.873	5.97	0.184
lima bean	3.36	1.66	11.4	0.363
dry milk, nonfat	5.08	2.40	16.4	0.375
soybean	8.26	3.59	24.6	0.558

[a] Adapted from ref 7. [b] See ref 252 for conversion factors of N to protein.

We found that (a) the lithium acetate−DMSO ninhydrin reagent gives consistently reproducible color yields using a range of structurally diverse proteins with low (wheat gluten) to high (casein, soybean protein) lysine content and a range of food flours with variable lysine content, (b) the net absorbance due to lysine was related to the lysine content independently determined by amino acid analysis, (c) the net absorbance per lysine residue in terms of Leu equivalents ranged from 0.638 for lactalbumin to 1.10 for bovine serum albumin (with an average and SD for this series of proteins of 0.825 ± 0.147), and (d) carbohydrates do not seem to interfere in the ninhydrin reaction of flours made from barley, corn, lima bean, soybean, rice, and wheat seeds and commercial milk powder (**Tables 4** and **5**).

The absorbance values for the ninhydrin reaction of food flours reflect the combined contributions to color yields for ϵ-NH_2 groups, N-terminal α-NH_2 groups, and NH_2 groups of any free amino acids present. To obtain the ninhydrin-reactive lysine content, the observed absorbance values need to be corrected for contributions of N-terminal NH_2 groups and for contributions by free amino acids. The latter correction requires an independent assay for free amino acids or removal of free amino acids prior to the ninhydrin assay for lysine (*178*−*182*).

Keratin Proteins. Human hair (*40*) and wool (*183*) are highly disulfide-cross-linked, insoluble proteins. In principle, the number of NH_2 groups to which dyes and other molecules can bind (*184*−*188*), even of an insoluble peptide such as wool or

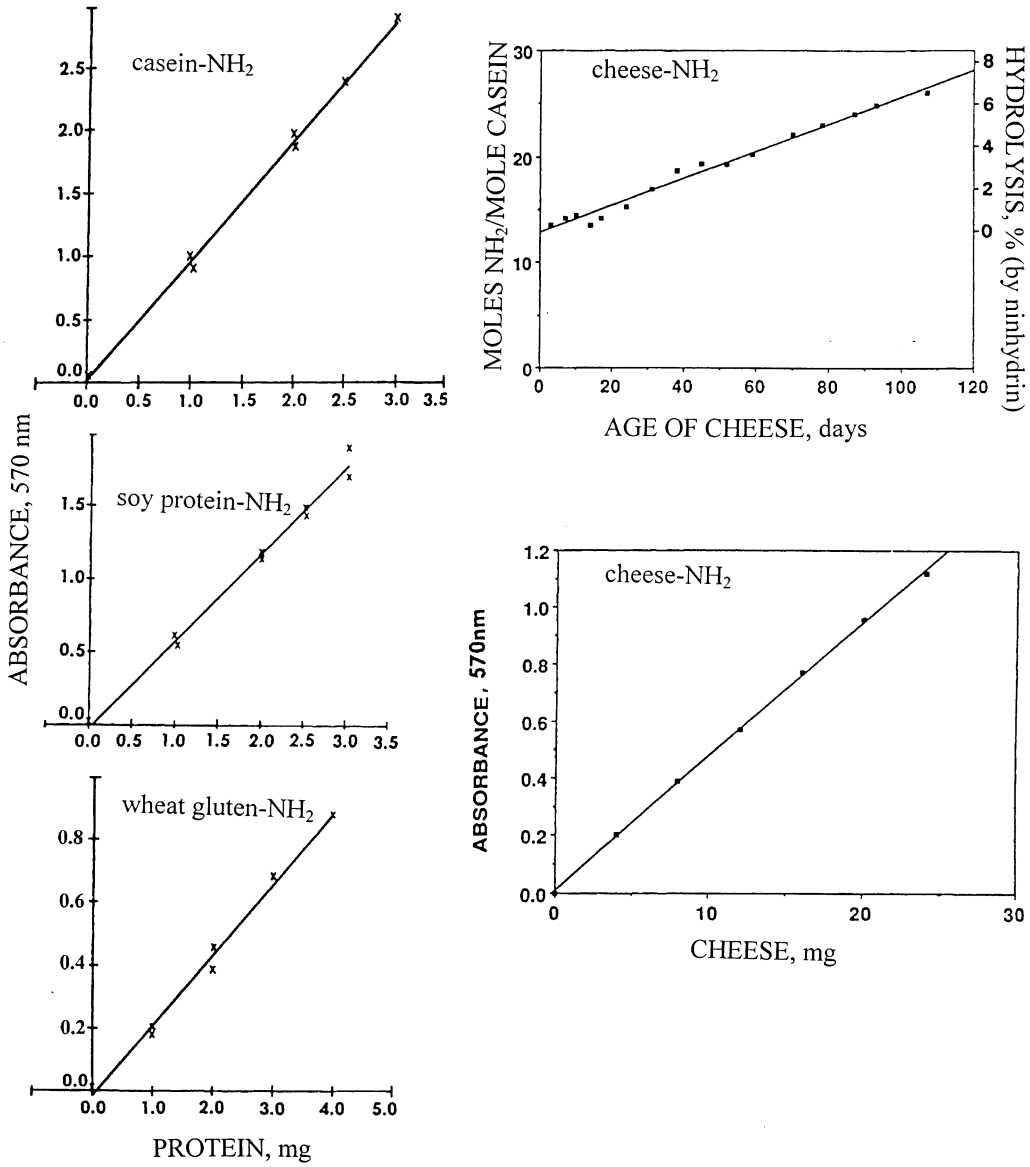

Figure 9. Linear relationships between ninhydrin color yields and concentrations of food proteins (*7*, *8*).

human hair, can be determined by reaction with ninhydrin and measurement of the amount of dye liberated in solution, without having to resort to dialysis, hydrolysis, or chromatography. The advantages of such a procedure would be the sensitivity of the ninhydrin reaction, its specificity, and its applicability without hydrolysis of the protein. The question is whether conditions can be found under which keratins can be analyzed similarly to soluble proteins to give a high, reproducible color yield.

The reaction of ninhydrin with several fibrous proteins such as wool, mohair, human hair, sheep's horn, feather, and silk was studied to determine conditions for optimum production of the colored ninhydrin derivative, RP (*5*). The best solvent system was found to be DMSO−water in the range 40−60% vol. Under optimum conditions, the ninhydrin color yield from keratin proteins is equivalent to that from soluble proteins. The ninhydrin assay is useful for estimating free NH_2 groups provided corrections are applied for extrapolation of the color yield back from long reaction times to zero time to remove contributions by the slow secondary reaction and for the 60−70% color yield from ϵ-NH_2 groups.

Silk, chicken feathers, and even sheep horn filings yield most of their color within 10−20 min; and like wool, all proteins studied seem to give a gradual rise in color yield after the major initial reaction. Extrapolating out this slow increase to zero times gives color yields equivalent to the lysine content for feather, mohair, and the wools. Silk and sheep horn give higher yields than expected, but in the case of silk, this is because the N-terminal amino group concentration is no longer negligible relative to the lysine content.

DMSO may exert its beneficial influence on the ninhydrin reaction of keratin proteins by (a) lowering the ionization of the amino groups, thus enhancing their rates; (b) swelling the keratin proteins, thus permitting better penetration of the ninhydrin reagent into highly disulfide-bond cross-linked keratin proteins; and (c) allowing the use of a stable, concentrated ninhydrin solution. DMSO also facilitates histochemical use of the ninhydrin reaction (see below).

Total Protein by Ninhydrin. Hydrolysis of a protein followed by amino acid analysis can in principle be used to measure the total protein content of a sample, provided the hydrolysate does not contain other ninhydrin-reactive or interfering compounds such as ammonia, free amino acids, amines, nucleic acids, carbohydrates (*146*, *160*, *161*) or cysteine (*189*). A method for the amino acid composition of β-lactoglobulin is based on analysis of its amino acid hydrolysate by paper chromatography (*89*). Another method is based on hydrolysis

Table 6. Ninhydrin Color Yields of Selected Fresh Fruits and Vegetables[a]

sample	Leu equivs[b]
red apples	6.2
pears	6.6
iceburg lettuce	7.6
avocado #l	9.3
green apples	10.2
avocado #2	14.6
redleaf lettuce	24.4
Romaine lettuce	24.7
Del Monte bananas	29.3
Dole bananas	32.6

[a] Procedure: The ninhydrin solution was prepared by dissolving ninhydrin (400 mg) and hydrindantin (60 mg) in a colored 100-mL volumetric flask, to which was added DMSO (15 mL) and 4 M lithium acetate buffer (5 mL). The suspension was stirred until all solid particles were dissolved. Samples (100–300 mg) were weighed into test tubes. Water (1 mL) and ninhydrin solution (2 mL) were added to each tube. The tubes were covered with 10-mL beakers, placed in a circular wire rack in a boiling water bath, covered with aluminum foil, and boiled for 30 min. They were then removed and placed in an ice water bath for 5 min. To each tube was added 50% ethanol (6 mL). The tubes were vortexed, centrifuged, and covered with Parafilm for 5 min. Leu standards, samples, and blanks were diluted with 50% ethanol to obtain readings in the As range of 0.4–0.7 at 570 nm. [b] Leu equivs ($\times 10^{-4}$) = A at 570 nm/mg of sample/A of 0.1 mg of Leu.

Table 7. Effect of Glucose on the Extent of Ninhydrin Color Formation of Casein and Soy Protein[a]

protein	conditions	λ_{max} at 570 nm/mg	% NH_2 modified
casein control		1.43	0.0
casein + 10% glucose	37 deg C, 10 days	0.93	35.0
casein + 10% glucose	95 deg C, 4 h	0.63	55.9
soy protein control		0.75	0.0
soy protein + 10% glucose	37 deg C, 10 days	0.46	38.3
soy protein + 10% glucose	95 deg C, 4 h	0.35	53.7

[a] Adapted from ref 19. Ninhydriun procedure: Lithium acetate buffer (4 M) was prepared by dissolving LiOH (168 g) in glacial acetic acid (300 mL) and H_2O (400 mL). The pH of this solution was adjusted to 5.2 with LiOH or acetic acid. The solution was brought to a volume of 1 L with H_2O. The ninhydrin was prepared by dissolving ninhydrin (2 g) and hydrindantin (0.3 g) in N_2-saturated DMSO (75 mL) in a dark glass container. To this solution was then added 4 M lithium acetate buffer (25 mL). The ninhydrin reaction was carried out by adding 2 mL of reagent to 1–3 mg of native or modified proteins in duplicate test tubes and to a tube without protein. The tubes were covered with glass beakers and placed in boiling water for 15 min. A 5-mL sample of 50% ethanol was then added to each cooled tube. The tubes were vortexed and the spectra determined. The ratio of the λ_{max} at 570 nm from treated and native proteins was used to estimate the extent of modification of NH_2 groups by glucose.

of 19 proteins by $Ba(OH)_2$ followed by analysis of the free amino acids with ninhydrin (*190*). Multilaboratory studies designed to optimize the hydrolysis and analysis of food proteins are described in (*38*, *39*, *82*).

Ninhydrin reactions were used in a number of related areas. These include (a) determining of the amino acid composition of stained proteins separated by SDS-gel electrophoresis (*191*) and (b) quantitating total protein based on the amino acid content of protein hydrolysates (*192*). The use of ethylene glycol as the solvent stabilized the ninhydrin reagent and did not require a nitrogen atmosphere during mixing and storage. All 18 proteins investigated contributed the same color intensity/μg of protein as did BSA. As mentioned earlier, ninhydrin is also stable when dissolved in a DMSO−lithium acetate buffer (*7*) as well as in 2% collidine−50% ethanol (*89*).

Fruits and Vegetables. We used a ninhydrin/chromogenic reaction to determine the contents of NH_2 groups in fruits and vegetables (*9*) (**Figure 8**, **Table 6**). In terms of Leu equivalents defined in **Table 6**, the values ranged from 6.2 for red apples to 32.6 for Dole bananas. This assay can be used to study the fate of NH_2 groups in amino acids, peptides, proteins, and in fruits and vegetables during ripening, browning, dehydration, and storage.

Screening Test for Whey in Milk. A screening test for the presence of added whey (a byproduct of cheese production) in milk is based on the acid ninhydrin reaction of *N*-acetylneuraminic (sialic) acid (470 nm) present in a glycopeptide of whey (*51*). The test was validated with 327 milk samples, 56 whey samples, and 70 milk samples "adulterated" with 5, 10, or 20% of whey. The ninhydrin-sialic acid chromophore is depicted in **Figure 3**.

Protein−Tannin Complexes. Special methods have been developed to measure total protein in situ (without extraction and without interference in the ninhydrin reaction by lignins and tannins) in 33 plant samples and six oak species (*48*−*50*). Unlike the ninhydrin method, the Lowry and Bradford methods were unsatisfactory for dried plant samples due to the incomplete extractability of the proteins and the sensitivity of both methods to the presence of tannins. An added advantage of the ninhydrin method is the direct estimation of proteins rather than from total *N* values. Measurement of protein−tannin complexes by ninhydrin provides tools for assessing the role of these complexes and of tannins in the plant and in the diet.

In a related study (*193*), removal of tannins from a hot water extract of tea leaves by complexation with poly(vinylpyrrolidone) facilitated the use of a method based on the ninhydrin reaction to determine 18 free amino acids in tea.

Other Applications to Proteins. Reported applications of the ninhydrin assay to proteins include (a) estimation of lysine in maize proteins and other cereal grains mentioned earlier (*178*−*182*); (b) assessment of the extent of modification in food proteins due to processing and the effect of these changes on protein utilization by ruminants (*194*−*196*); and (c) measurement of the extent of alkylation (**Figure 7C**), acetylation (**Figure 8**), and glucosylation (**Table 7**) of protein amino groups (*19*, *22*, *42*−*45*, *197*, *198*). As already mentioned, because the method is sensitive to changes in the free amino group content of a protein, it is especially useful for following the extent of chemical modifications resulting from protein-carbohydrate (Maillard) reactions.

Protein Kjeldahl Nitrogen by Ninhydrin. A highly sensitive automated assay of Kjeldahl N using a ninhydrin reagent is based on the reduction of hydrazine to hyndrindantin (*81*). Application of the assay to 28 wheat samples as well as to rapeseed flour and to soy protein concentrates digested with H_2SO_4 showed good agreement with values obtained by a standard micro-Kjeldahl assay for N.

PROTEOLYSIS OF PEPTIDE BONDS

Enzyme-Catalyzed Cheese Ripening. Cheeses are stored before consumption to allow the cheese to ripen and to develop its characteristic flavor and texture. Endogenous milk proteases such as plasmin as well as microbial enzymes and enzymes used to coagulate the milk (the rennets) act in concert to break down the protein in the cheese (*199*). This generally leads to the development of both desirable and undesirable characteristics in the cheese. Proteolysis results in the formation of new amino groups. Because we found that a stable ninhydrin reagent containing lithium acetate and DMSO, used in the lysine assay

Table 8. Concentration of Free Amino Groups in Various Cheeses as Determined by a Manual Ninhydrin Assay[a]

cheese	λ_{max} at 570 nm	protein (%)	moles NH_2/ mole protein
teleme	0.399	20	12.6
cheddar (3 days old)	0.432	22.2	12.8
feta	0.419	20	13.4
Parmesan	0.644	33	17.5
Monterey Jack	0.568	20	17.7
cheddar (3 months old)	0.914	22.2	26.0
brie	0.753	20	34.1
Gouda	0.804	20	36.2

[a] Adapted from ref 8.

described earlier, is a good solvent of foods such as cheese, we investigated the application of the ninhydrin reaction to the study of cheese ripening (*8*). Ideally, all cheese protein, peptide, and amino acid N-terminal amino groups would have the same color yield. However, amino acids and small peptides differ somewhat in their color yields when reacting with ninhydrin, presumably because of slow or incomplete reaction or the formation of byproducts (**Table 1**). The most notable case of this is proline, which contains no primary amino group and constitutes about 12 mol % of casein, the major protein of cheese. As the cheese ripened, there was a progressive and steady increase in free amino groups (**Figure 9**, **Table 8**).

More than 700 unique peptide bonds are possible in this protein mixture; thus the number of possible proteolysis products is very large. However, only a limited number of peptides are formed. The same chromophore, RP, is produced for all primary amines that react with ninhydrin. This chromophore is fully soluble under the conditions of the assay. It is not chemically bound to the protein and is, therefore, not lost when insoluble protein is removed by centrifugation. Its color is distinctive, and there is no interference from yellow chromophores, common in many foods. Light scattering and turbidity values are lower at 570 nm than at shorter wavelengths. These considerations suggest that the ninhydrin assay has major advantages over other procedures, which are based on the direct attachment of a chromophore to the casein.

The proteolysis of cheese proteins can also be followed with the aid of a Cd-ninhydrin reagent (*103*, *200*). Biogenic amines in cheese can be analyzed by HPLC using a ninhydrin-containing eluent (*173*), thus eliminating the need for postcolumn derivatization.

In summary, the ninhydrin chromogenic reaction has been used to determine the concentration of free amino groups in ripening cheeses. This analysis involves dispersing cheese in citrate solution, heating a sample of the resulting mixture with aqueous DMSO−ninhydrin plus lithium acetate at pH 5.2, and determining the absorbance. The method appears applicable to diverse cheese varieties. The assay merits further exploration as a general method for measuring proteolysis and digestibility of foods.

Proteolysis by Ruminant Microbial Enzymes. Protein nutrition of ruminants (cattle, sheep) depends on their ability to degrade and synthesize feed proteins (*201*). Ruminal protein degradation consists of microbial enzyme-catalyzed peptide bond cleavage and deamidation of amide residues to NH_3. As part of an effort to improve ruminant nutrition, Broderick and Kang (*194*) developed an automated ninhydrin assay for the rapid estimation of the extent of both proteolysis and NH_3 formation in ruminant fluids. The method permits assessment of the nutritional quality of native and modified proteins consumed by ruminants (*196*, *202*−*204*).

Alkali-Induced Proteolysis. We studied the effect of alkali-induced peptide bond cleavage by a ninhydrin assay and concurrent racemization of amino acid residues of eight proteins (*24*, *41*, *197*). The extent of proteolysis was measured in terms of Leu equivalents, defined as follows: Leu equivalent = As_{570} per mg protein N/(As_{570} per mg Leu). The susceptibility of peptide bonds to alkali-induced proteolysis varied widely among the structurally different proteins. In addition, the extent of peptide bond cleavage affected the susceptibility of L-amino acid residues to racemization to D-isomers.

Related Proteolysis Studies. The following are some additional applications of the ninhydrin assay to assessments of peptide bond hydrolysis: (a) estimation of proteolysis of senescent rice leaves (*205*) and of pea (*Pisum sativum*) ovaries (*206*), (b) proteolysis in meat (*207*) and processed food proteins (*208*), (c) proteolytic activities of peptidases (*209*), (d) monitoring of NH_2 groups in peptides by HPLC (*210*), (e) cleavage of peptide bonds by chymosin (rennin) (*211*), and (f) gelatin degradation at elevated temperatures (*212*).

FORENSIC AND BIOMEDICAL SCIENCES

The following additional applications demonstrate the adaptability of the ninhydrin reaction to the solution of problems in criminalistics, histochemistry, and microbiology.

Forensic Science. Ninhydrin has become the universal reagent for the chemical development of latent fingerprints on porous surfaces such as paper (*99*, *100*, *213*−*215*). Extensive efforts have been made to enhance the usefulness of the ninhydrin method in forensic science. These include (a) sensitivity enhancement of ninhydrin-treated latent fingerprints by proteolytic enzymes (trypsin, chymotrypsin) that increase the amount of free amino acids in palm respiration and (b) formation of metal (cadmium, europium, iron, zinc) ion complexes between RP and a variety of metal salts (**Figure 5**). These complexes fluoresce strongly under the blue-green light of an argon laser. For example, the RP/$ZnCl_2$ fluorescent and the RP/$Cd(NO_3)_3$ photoluminescent methods (*216*) develop fingerprints in detail, where none are visible with ninhydrin alone under blue light (*217*).

Histochemistry. Because the ninhydrin reaction results in decarboxylative deamination of amino acids to an aldehyde, it was possible to use the reaction cytochemically, whereby the in situ conversion of amino to aldehyde groups combined with a so-called Schiff's reagent (acriflavin, fuchsin) would form a colored stain. Tissue amino groups producing reactive aldehydes are those associated with free amino acids, N-terminal α-amino acids, and ϵ-NH_2 groups of lysine residues (*218*). The discovery of the ninhydrin−Schiff staining technique stimulated widespread interest in applying the method to histochemistry. Examples include studies of (a) a ninhydrin-specific chemical test for hair keratin (*219*), (b) energy and amino acid metabolism in mouse brain (*220*), (c) protein and DNA content of cells (*221*), (d) the cytochemistry of hepatic lysosomes and their enzymatic aggregates by a ninhydrin-DMSO-thiosemicabazide silver protein reaction (*222*), and (d) the use of a ninhydrin reagent to assess histochemically protein-bound amino acids in rat tissues (*223*). The DMSO−H_2O mixed solvent enhanced the rate of oxidative deamination and favored specific staining of tissues (*222*).

Microbiology and Medicine. The ninhydrin reaction facilitated the analysis, isolation, and characterization of numerous antibiotics, bacterial toxins, and microbial products containing

ninhydrin-reactive amino groups. Studies in this area include (a) photometric analysis of the antibiotic cefalotin with an acid ninhydrin reagent (*224*), (b) isolation of a toxin from *Bordetella pertussis* (*225*), (c) purification of the antibiotic microcin (*226*), (d) determination of the antibiotics gentamycin and streptomycin by chromatography with a mobile phase containing ninhydrin as a fluorogenic reagent (*227*−*229*), (e) determination of amino glycoside antibiotics (*230*), (f) characterization of bacereutin, an antifungal antibiotic produced by *Bacillus cereus* (*231*), (g) determination of the antibiotic neomycin in ophthalmic ointments (*232*), (h) analysis of the antibiotic cephalosporin in pharmaceutical samples (*233*), (i) binding of phosphatidylethanolamine to *Actinobacillus pleuropneumoniae* (*234*), (j) analysis of glycine formed during the hydrolysis of hippurate to identify *Campylobacter* microorganisms (*235*), (k) analysis of bacterial infection of plant nodules by *Rhizobium loti* (*236a*), and the transformation of compost components (*236b*).

Other applications to medicinal chemistry and medicine include (a) measurement of O-phosphohydroxylysine in patients with neurological abnormalities (*237*), (b) analysis of epileptogenic guanidine compounds in the brain (*238*), (c) early detection of leprosy (*106*), (d) analysis of the antiulcer drug famotidine (*239a*), anti-osteoporosis drugs (*239b*), and of the drug gabapentin used to treat neuralgia (*104*), and (e) assessment of the role collagen in artificial cartilage tissue (*240*).

Cyanide reacts with ninhydrin in alkaline solution to form a blue-colored product with a λ_{max} of 590 nm (*101*). This reaction can be used to measure low levels of cyanide in industrial effluents. These studies demonstrate the wide-ranging benefits of the ninhydrin reaction to microbiology, medicine, and environmental toxicology.

PHARMACOLOGY AND TOXICOLOGY OF NINHYDRIN

Ninhydrin is reported to induce both adverse and beneficial effects when ingested by laboratory animals as well as in cell cultures, as illustrated by the following observations. It is a strong stage I tumor promoter in the multistage mouse skin carcinogenesis assay, but did not appear to induce tumorigenesis when tested as a stage II or as a complete tumor promoter (*241*). Tumor promotion was accompanied by enhanced activity of the enzyme γ-glutamyl transpeptidase. By contrast, ninhydrin acted as an anti-carcinogen in Ehrlich ascites carcinoma cells (*242*). The antitumor activity of ninhydrin was similar to that of cyclophosphamide. Ninhydrin protected against ethanol-induced damage of the gastric mucosa of rats (*243*). The anti-ulcerogenic activity of ninhydrin appears to be due to its ability to oxidize mucosal SH groups to disulfide bonds. Although not diabetogenic, ninhydrin was toxic to rat pancreatic beta cells at low concentration, destroying all islet cells at high concentrations (*244*). Glucose did not protect the pancreatic cells against ninhydrin but did protect against the diabetogenic action of alloxan, a compound structurally related to ninhydrin. Both compounds inhibited the enzyme glucokinase in the pancreatic cells, presumably by oxidation of the essential SH groups of the enzyme (*245*). Ninhydrin appears to destroy the pancreatic cells before it can induce diabetes.

Other studies report that ninhydrin down-regulated antioxidative enzymes (catalase, glutathione peroxidase, superoxide disumutase) in pancreatic cells (*108*), inhibited the enzyme aconitase in liver cells (*245*), and induced neurological changes in rodents.

The cited observations suggest that laboratory workers and others should avoid exposure to ninhydrin. Whether this is also true for Ruhemann's purple is not known.

CONCLUSIONS AND OUTLOOK

The chemistry and applications of ninhydrin reactions with a variety of substrates has undergone and continues to undergo major developments over the course of nearly 100 years. A major objective of this review has been to interest investigators to look at the developments beyond their area of expertise where developments have occurred with which they may not be familiar. For example, other disciplines have apparently not adopted the exciting developments in the use of the reaction in forensic science, which take advantage of the fact that RP forms complexes with a of number of metal ions that can be detected at extremely low concentrations by fluorescent, phosphorescent, and luminescent techniques. These methods merit further development and application to the analysis of amino, peptides, and proteins. Ninhydrin assays of high sensitivity could be helpful in the currently evolving field of proteomics, which attempts to account for all individual proteins in a cell, requiring analytical tools with high sensitivity. Another area in which the ninhydrin reaction merits wider use is in both classical and molecular biology plant breeding programs, which aim to increase both quantity and quality of high-protein, high-lysine, and high-methionine edible proteins needed to feed the ever-growing human population (*247*−*249*).

So-called "nonclassical" ninhydrin reactions, which do not involve formation of RP, are also being adapted to practical use. Examples include the use of acid ninhydrin reagents to measure cysteine, protein-bound tryptophan, pipecolic acid, and sialic acid as well as the reaction of ninhydrin with cyanide ions under alkaline conditions to form a chromophore whose concentration gives a direct measure of cyanide ion content of polluted waters. The behavior of unnatural amino acids derived from plant sources (*83*, *250*) in the ninhydrin reaction also merits study. Finally, an important objective of research is to more fully understand the underlying chemistry upon which numerous applications are based. Such an understanding will hopefully lead to improvements in the application of ninhydrin reactions to the solution of problems in agricultural, food, and protein chemistry and a wide range of related sciences.

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